IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Masayori Inouye	**Examiner:**	Catherine Hibbert
Application No.:	10/560,303	**Confirmation No.:**	9634
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DECLARATION OF DR. KIYOYUKI MATSUMURA

I, the undersigned, hereby declare and state that:

1. I am an employee of Takara Bio Inc., located at Seta 3-4-1, Otsu, Shiga 520-2193, Japan. I received a doctorate degree in science from the University of Tokyo in 1995. I have engaged in research and development relating to molecular biology with Takara Bio Inc. since 2008.

2. I understand that the University of Medicine and Dentistry of New Jersey ("UMDNJ") is the assignee of U.S. Application Serial No. 10/560,303, filed March 29, 2007 (the "Application"). I also understand that Takara Bio Inc. has licensed the rights to the Application from UMDNJ.

3. I have reviewed the amended claim set which is being presented concurrently with this supporting declaration (the "Claims") and I understand the scope of Claims, particularly with respect to the type of cells used in making polypeptides (i.e., prokaryotic cells) and the growth conditions under which the polypeptides are made (i.e., any growth media).

4. I have reviewed the Office Action mailed May 23, 2011 (the "Action"). In the Action, at page 3, the Examiner rejected claims 24, 27-31 and 36-38 of the Application under 35 U.S.C. § 112, first paragraph. I understand that the Examiner takes the position that "because the specification, while being enabled for a method of making a polypeptide using the cell strains, substrates, and growth conditions as shown in the instant specification under Example IV, does not reasonably provide enablement for a method of making a polypeptide using any cell types under any growth conditions as now claimed".

5. From the Application's teaching, a polypeptide can be made in a cell by (i) transfecting the cell with a mutated nucleic acid; (ii) transfecting cell with an mRNA interferase; and (iii) expressing the nucleic acids of the polypeptide and the mRNA interferase. The Application exemplifies polypeptide synthesis in Example VI, beginning at page 113 of the Application.

6. Example VI of the Application teaches the following:

> *E. coli BL21(DE3) comprising pACYCmazF or BL21(DE3) comprising pACYCmazF and pCold(SP)eotaxin were grown in M9-glucose medium containing appropriate antibiotics. When the OD_{600} of the culture reached 0.5, the culture was shifted to 15°C for 15 minutes and 1 mM IPTG was added to the culture. At the indicated time intervals, 1 ml of culture was removed and added to a test tube containing 10 μCi ^{35}S-methionine. After incubation for 15 minutes (pulse), 0.2 ml of 50 mg/ml methionine was added and incubated for 5 minutes (chase). The labeled cells were washed with M9-glucose medium and suspended in 100 μl of SDS-PAGE loading buffer. 10 μl of each sample was analyzed by SDS-PAGE followed by autoradiography.*
>
> *pCOLDI comprising a novel nucleic acid sequence encoding mature human eotaxin was used as a template. See FIG. 36. The resultant plasmid was designated pCold(SP)eotaxin. Plasmid pACYCmazF comprising a mazF gene under the control of a T7 promoter was used for inducing expression of MazF. E. coli BL21(DE3) cells comprising either pACYCmazF alone or pACYCmazF and pCold(SP)eotaxin were grown in M9-glucose medium containing appropriate antibiotics. When the OD_{600} of the culture reached 0.5, the culture was shifted to*

> *15°C for 15 minutes and 1 mM of IPTG was added to the culture. At the indicated time intervals, 1 ml of culture was added to a test tube containing 10 μCi ^{35}S-methionine. After a 15 minute incubation in the presence of label (pulse), 0.2 ml of 50 mg/ml methionine was added and the culture incubated for 5 minutes (chase). The labeled cells were washed with M9-glucose medium and suspended in 1001 of SDS-PAGE loading buffer. 10 μl of each sample was analyzed by SDS-PAGE followed by autoradiography. Expression of the mazF gene inhibited the protein synthesis in BL21(DE3) cells, as reported previously (Zhang et al. 2003, supra). Synthesis of mature human eotaxin, however, which is encoded by an mRNA which does not comprise an ACA sequence, was not inhibited by mazF expression. See FIG. 37. This result demonstrates that large quantities of a single protein can be obtained using the single-protein production method of the present invention.*

7. I understand that, as recited in paragraph 6 above, Example VI of the Application exemplifies polypeptide synthesis using *E. coli* as the cell type, and minimal media (i.e., M9-glucose) as the growth medium.

8. It is my position that, from the Application's teachings, a skilled artisan would understand that any prokaryotic cell can be used to synthesize polypeptides. A skilled artisan would also recognize that different growth media can be used, and based on common knowledge in the art, selecting the appropriate growth media would not involve anything more than routine experimentation.

9. Using the methods taught by the Application, and specifically Example VI, I successfully synthesized a polypeptide using a different prokaryotic cell (i.e., *B. subtilis*) using a rich medium (i.e. LB medium). The details of the synthesis are as follows:

10. Construction of the *Bacillus subtilis* strain harboring MazF gene. Tet gene, which encodes tetracycline efflux protein, was isolated from plasmid pHY300PLK (manufactured by Takara Bio Inc.) and introduced into MazF expression plasmid, pMazF (included in "SPP System I"; manufactured by Takara Bio Inc.). The plasmid thus obtained was named pMazF-tet.

11. A DNA fragment containing a constitutive promoter derived from *veg* gene of *Bacillus subtilis* was amplified by PCR from the genomic DNA of *B. subtilis*. The primers used in this PCR were prepared based on the genomic sequence information of *Bacillus subtilis* published at GenBank under an accession No. NC_000964. The *lacI* promoter located at the upstream of *lacI* coding region in plasmid pMazF-tet was replaced with the DNA fragment amplified and plasmid pMazF-veg-tet was obtained.

12. An artificial promoter having the nucleotide sequence represented by SEQ ID NO: 1 (shown on Appendix A) was constructed by adding the nucleotide sequence of *lac* operator and the recognition sequence of catabolite control protein A (CcpA) to grac promoter shown in Phan *et al.* (Protein Expression and Purification, 2006, Vol. 46, pages 189-195) for an inducible expression in *B. subtilis*. The *lac* promoter in plasmid pMazF-veg-tet was replaced with this artificial promoter, and the obtained plasmid was named pN-veg-glac-c.

13. *B. subtilis* strain RIK1285 (included in "B. subtilis Secretory Protein Expression System"; manufactured by Takara Bio Inc.) was transformed by plasmid pN-veg-glac-c with the method described in the manual of *B. subtilis* Secretory Protein Expression System. Several transformants were isolated after overnight cultivation on the LB agar plate containing 10 μg/ml of tetracycline. Among them, a strain of which growth was repressed on the LB agar plate containing 10 μg/ml of tetracycline and 1 mM of IPTG was chosen, and it was named *B. subtilis* strain Nvc.

14. Construction of the plasmid for expression of beta-lactamase. An artificial polynucleotide having the nucleotide sequence represented by SEQ ID NO: 2 (shown on Appendix A) was synthesized. It contains the promoter sequence and the coding region for signal peptide derived from *aprE* gene of *B. subtilis*, multi-cloning site of plasmid pCold I

(manufactured by Takara Bio Inc.), the coding region for His-Tag peptide and the transcriptional terminator sequence derived from plasmid pCold I. ACA triplet sequences in the coding region for AprE signal peptide were replaced with alternative triplet sequence without altering the encoded amino acid sequence.

15. An artificial polynucleotide having the nucleotide sequence represented by SEQ ID NO: 3 (shown on Appendix A) was also synthesized. It encodes the amino acid sequence of beta-lactamase derived from *Escherichia coli* but does not contain ACA triplet sequence.

16. The region spanning *aprE* promoter to multi-cloning site of plasmid pBE-S (included in "*B. subtilis* Secretory Protein Expression System"; manufactured by Takara Bio Inc.) was replaced with the artificial polynucleotide of SEQ ID NO: 2, and then, the artificial polynucleotide of SEQ ID NO: 3 was introduced into the multi-cloning site of the obtained plasmid so that beta-lactamase having AprE signal peptide and His-Tag can be expressed under the control of *aprE* promoter. The obtained plasmid was named pLac-His.

17. Expression of beta-lactamase in *B. subtilis*. *B. subtilis* strain Nvc and strain RIK1285 were transformed by pLac-His, respectively. The transformants obtained were cultivated in 2 ml of KT-LB medium (LB medium containing 10 μg/ml of kanamycin and 5 μg/ml of tetracycline) at 28° overnight. 0.2 ml portion of each culture broth were added 3.8 ml of KT-LB medium and KT-LB medium containing 1 μM IPTG, respectively. The inoculated media were cultivated at 37° or 28°, and aliquots of each culture broth were took out after 5, 10 or 28 hours after the beginning of the cultivation. The proteins contained in the supernatant of the culture broth were analyzed by SDS-PAGE. The result was shown in Figure 1, attached herewith as Appendix B. As shown in Figure 1, many kinds of proteins as well as beta-lactamase were secreted into the culture broth of strain RIK1285 harboring pLac-His. On the

contrary, the proteins other than beta-lactamase were significantly decreased in the culture broths of the transformants derived from strain Nvc as a host. In KT-LB medium containing 1 μM IPTG, the proteins other than beta-lactamase were further decreased.

18. *B. subtilis* strain Nvc and strain RIK1285, which were both harboring pLac-His, were cultivated in 2 ml of KT-LB medium at 28° for 28 hours. The supernatants were collected from each culture broths, and the low molecular weight proteins were removed from 400 μl of each supernatant by repeating the concentration using ultrafiltration membrane and the dilution with phosphate-buffered saline, for 3 times. Finally, the supernatants were concentrated to 40 μl, and beta-lactamase activities in concentrated supernatants were assayed by the method of Waley (Biochemical J., 1974, Vol. 139, pages 789-790). The beta-lactamase activity in the culture broths of strain Nvc harboring pLac-His and strain RIK1285 harboring pLac-His were 7.2 U/μl and 0.34 U/μl, respectively. The specific activities of beta-lactamase in each culture broths calculated from these values were 61 U/mg protein and 2.4 U/mg protein, respectively.

19. For the reasons set forth above in paragraphs 5-18, the use of any prokaryotic cell and any growth media are supported by the Application.

20. I hereby declare further that all statements made herein by our own knowledge are true and that all statements made on information and belief are believed to be true and further that we make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent issuing therein.

Signed this 15 day of September, 2011

Kiyoyuki Matsumura

Dr. Kiyoyuki Matsumura

APPENDIX A

Sequence Listing

SEQ ID NO: 1

AGATCTGGCA GTGAGCGCAA CGCAATTGAA AAGAATGATG TAAGCGTGAA AAATTTTTTA 60
TCTTATCACT TGAAATTGAA AGCGCTTTCA TTTATTATAA GAATTGTGGA ATTGTGAGCG 120
GATAACAATT CCCAATTAAA GGAGGAAGGA CATATG 156

SEQ ID NO: 2

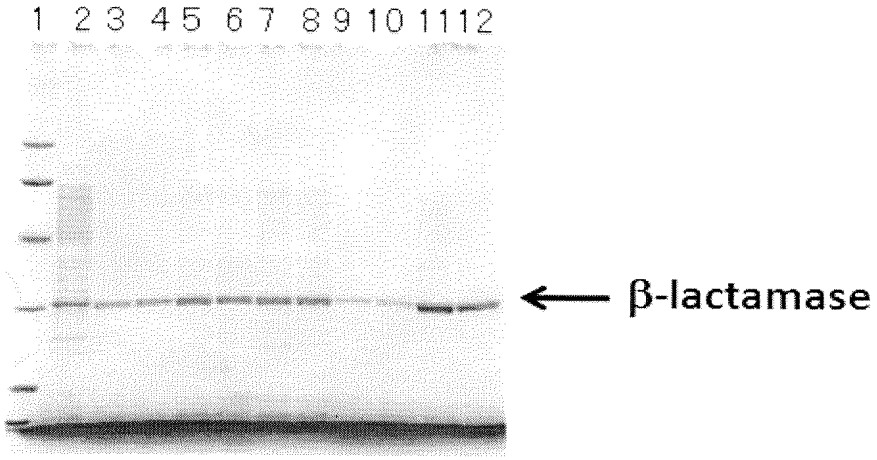
ACTAGTGTTC TTTTCTGTAT GAAAATAGTT ATTTCGAGTC TCTACGGAAA TAGCGAGAGA 60
TGATATACCT AAATAGAGAT AAAATCATCT CAAAAAAATG GGTCTACTAA AATATTATTC 120
CATCTATTAT AATAAATTCA GAGAATAGTC TTTTAAGTAA GTCTACTCTG AACTTAAGCA 180
AAAGGAGAGG GACGCGTATG AGATCAAAAA AACTGTGGAT TTCACTGCTG TTTGCTCTGA 240
CGCTGATTTT TACGATGGCG TTTAGCAATA TGTCAGCGCA AGCAGCGGCC GGTGCGCATA 300
TGGAGCTCGG TACCCTCGAG GGATCCGAAT TCAAGCTTGT CGACCTGCAG TCTAGAGCGC 360
ATCATCATCA TCATCATTAA CTGTCAGACC AAGTTTACTC ATATATACTT CCCTGCCATT 420
TGGCGGGGAT TTTTTTATTT GTTTTATCGA TGCTAGC 456

SEQ ID NO: 3

CATATGCACC CAGAAACGCT GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA 60
GTGGGTTACA TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA 120
GAACGTTTTC CAATGATGAG CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT 180
ATTGACGCCG GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT 240
GAGTACTCAC CAGTCACAGA AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC 300
AGTGCTGCCA TAACCATGAG TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA 360
GGACCGAAGG AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT 420
CGTTGGGAAC CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCT 480
GTAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC TCTAGCTTCC 540
CGGCAACAAT TAATAGACTG GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG 600
GCCCTTCCGG CTGGCTGGTT TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC 660
GGTATCATTG CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG 720
ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA 780
CTGATTAAGC ATTGGTCTAG A 801

APPENDIX B

Figure 1



Lane 1; Low range molecular weight marker (manufactured by Bio-Rad Laboratories, Inc.
Lane 2; strain RIK1285 harboring pLac-His, at 37□ for 10 hours in KT-LB
Lane 3; strain Nvc harboring pLac-His, at 37□ for 5 hours in KT-LB
Lane 4; strain Nvc harboring pLac-His, at 37□ for 5 hours in KT-LB containing 1 μM IPTG
Lane 5; strain Nvc harboring pLac-His, at 37□ for 10 hours in KT-LB
Lane 6 strain Nvc harboring pLac-His, at 37□ for 10 hours in KT-LB containing 1 μM IPTG
Lane 7; strain Nvc harboring pLac-His, at 37□ for 28 hours in KT-LB
Lane 8; strain Nvc harboring pLac-His, at 37□ for 28 hours in KT-LB containing 1 μM IPTG
Lane 9; strain Nvc harboring pLac-His, at 28□ for 10 hours in KT-LB
Lane 10; strain Nvc harboring pLac-His Nvc, at 28□ for 10 hours in KT-LB containing 1 μM IPTG
Lane 11; strain Nvc harboring pLac-His, at 28□ for 28 hours in KT-LB
Lane 12; strain Nvc harboring pLac-His, at 28□ for 28 hours in KT-LB containing 1 μM IPTG